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Research article

Copper excess detoxification is mediated by a coordinated and complementary induction of glutathione, phytochelatins and metallothioneins in the green seaweed *Ulva compressa*

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ABSTRACT

In order to analyze the involvement of intracellular thiol-chelators in the accumulation and detoxification of copper, the marine alga *Ulva compressa* was cultivated with increasing concentrations of copper such as 2.5, 5, 7.5 and 10 μM for up to 12 d, and the amount of intracellular copper, glutathione (GSH), phytochelatins (PCs) and transcripts encoding three metallothioneins (MTs) were determined. Over this exposure period and concentration range there was a linear correlation between intracellular copper and the copper concentration in the culture medium. Increases in GSH concentrations occurred mainly between days 1 and 3 and at lower concentrations of copper (2.5 and 5 μM). The level of PCs, and particularly PC2, increased from day 1 of exposure mainly at higher concentrations of copper (7.5 and 10 μM). The levels of transcripts encoding MT7 increased at day 3, whereas those of MT3 and MT6 increased between days 9–12, mainly at higher concentrations of copper. Thus in *U. compressa*, the initial responses to increasing intracellular copper concentrations are increases in GSH and PCs that are followed by higher levels of MTs expression, suggesting that thiol-containing peptides and proteins may participate in copper accumulation and detoxification responding in a coordinated and complementary manner. In addition, the alga was cultivated with 10 μM copper for 5 d and transferred to synthetic seawater with no copper and cultivated for 3 d. The release of copper from cells to culture medium was observed and accompanied by a similar nanomolar amount of GSH; no PCs or small proteins were detected. These results could suggest that a component of the detoxification mechanism also involves the release of copper and GSH to the extracellular medium.

1. Introduction

Metals such as copper, zinc, iron and manganese are essential elements for the metabolism of chloroxygenic organisms, playing important roles in the functioning of proteins required in photosynthesis (plastocyanin) and respiration (cytochrome c); also in enzymes such as cytochrome c oxidase, copper/zinc superoxide dismutase and some oxidases and dehydrogenases (Burkhead et al., 2009; Bothe, 2011; Singh et al., 2016). In this sense, it has been shown that plants cultivated with low concentrations of copper displayed a preferential allocation of copper to plastocyanin in chloroplasts of young leaves, compared to copper/zinc superoxide dismutase, metallochaperone CCH and polyphenol oxidase (Ravet et al., 2011). However, in excess these metals and other non-essential metals (e.g. lead, cadmium, chromium and others) are toxic and can induce oxidative stress and damage macromolecules (Singh et al., 2016; Fryzova et al., 2017). To counter the build-up of toxic metals at sensitive sites within cells, algae and plants use various mechanisms for homeostasis and detoxification. One such mechanism is the binding of metals to thiol-containing ligands and their subsequent compartmentalization. Two of the best-characterized intracellular thiol-chelators are phytochelatins and metallothioneins (Yadav, 2010; Fryzova et al., 2017). In addition, it has been described that micro and macroalgae can release cysteine and/or reduced glutathione (GSH) in response to copper, cadmium and lead stress (e.g.

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Leal et al., 1999, Vasconcelos and Leal, 2001a, b) suggesting that these thiol-containing molecules can complex metals in the extracellular medium to reduce their bioavailability and capacity to cross the cell wall and membrane. Thus, metal detoxification involves a combination of both extra- and intracellular mechanisms, their relative contribution apparently varying between species, between populations of a species, and seasonally (Villares et al., 2002; Malea et al., 2015; Moenne et al., 2016).

Phytochelatins (PCs) are small cysteine-rich polypeptides having the general structure: $(\gamma\text{-Glu-Cys})_n$-Gly and chain-lengths of between 2 and 11 units and chelation of metal is via sulphydryl groups (Cobbett and Goldsbrough, 2002). PCs are synthesized by the enzyme phytochelatin synthase (PCS) using GSH as substrate. Its activation occurs in the presence of metal ions or on their binding to GSH-complexes (Cobbett, 2000; Vatamaniuk et al., 2000; Osaki et al., 2009). Therefore, maintaining the equilibrium between synthesis and utilization of GSH and production of PCs may be critical to countering metal stress. While there is ample evidence for the formation of PC-cadmium and PC-arsenic complexes and their sequestration in the vacuole (Song et al., 2014; Zhang et al., 2018), similar involvement with other metals, such as copper, is less clear (Cobbett and Goldsbrough, 2002). For example, Mendoza-Cozatl et al. (2010), Song et al. (2010) and Song et al. (2014) provide evidence for the transportation of arsenic, copper, zinc and manganese bound to PCs to the vacuole by mean of ABCC-type transporters that require magnesium and ATP for their activity. However, while copper is a strong activator of PC synthesis and PC-copper complexes can form *in vitro*, a PC-deficient mutant of *Arabidopsis* displayed little sensitivity to copper exposure (Lee and Kang, 2005). Results from this study, and from a more recent one by Flores-Cáceres et al. (2015) that found no production of PCs in *Medicago sativa* under copper stress, suggesting that PCs are of less importance in the detoxification of copper ions. PCs-copper complexes may be transitory, only partially sequestered in the vacuole and therefore a more efficient detoxification mechanism may be functioning (Cobbett and Goldsbrough, 2002). One possible alternative involves the production of metallothioneins (MTs) which are low molecular weight, gene encoded cysteine-rich, metal-binding proteins that are found in many groups of organisms, including algae and plants (Cobbett and Goldsbrough, 2002; Morris et al., 1999). It has been proposed that MTs mediate metal accumulation, distribution and tolerance in plant/algal cells, although the evidence is quite limited. However, the results from several recent studies using transgenic *Arabidopsis* lend support for the latter response. For example, *A. thaliana* plants overexpressing MTs genes from *Brassica campestris*, *Oryza sativa* and *Iris lactea* var. *chinensis* displayed greater tolerance to cadmium and copper stress (Lv et al., 2013; Liu et al., 2015; Gu et al., 2018).

Marine macroalgae are considered as effective metal accumulator, although in comparison to terrestrial plants, less is known about their responses and mechanisms to thrive under metal excess. One of the best study models is the green macroalga *Ulva compressa,* and mechanisms involved in copper tolerance (Moenne et al., 2016). It was initially observed that *U. compressa* is the dominant species in highly copper-polluted sites of northern Chile, suggesting that the development of advances biological strategies to counteract copper stress in this alga (Castilla, 1996). Different investigations on the species have shown that its main mechanisms to withstand copper excess are based on cellular exclusion efforts, antioxidant defenses, and syntheses of metal chelators; however, the dynamics in the production of the different chelating molecules and their overall contribution to copper accumulation is still uncertain (Moenne et al., 2016). It was further determined that *U. compressa* cultivated *in vitro* can survive copper concentrations of up to 50 μM for 7 days (González et al., 2010). Most *in vitro* studies on the species have been performed using 10 μM copper, which is a realistic concentration considering the highest levels recorded in higher copper-polluted areas where *U. compressa* is found (Correa et al., 1999; Ratkevicius et al., 2003).

Although limited, there is evidence for metal (cadmiun, copper)-induced PCs production in green, red and brown seaweeds from both laboratory (e.g. Hu and Wu, 1998; Mellado et al., 2012; Roncarati et al., 2015) and field studies (Pawlik-Skowronska et al., 2007). For example, in *E. siliculosus* exposure to Cd induced PC2, PC3 and PC4, while for copper only PC2 and PC3 were produced (Roncarati et al., 2015). In contrast, exposure of the green seaweed *Ulva compressa* to 10 μM Cu for 7 d induced PC2, PC3 and PC4 (Mellado et al., 2012). Reports of the possible involvement of MTs in metal detoxification come from a few studies with brown and green seaweeds. For example, there was induced expression of a complete MT cDNA cloned in the brown macroalga *Fucus vesiculosus* on exposure to excess Cu (Morris et al., 1999). More recently, from a whole transcriptome analysis of *U. compressa* grown under 10 μM copper for 24 h, MTs transcripts increased on exposure to 10 μM Cu for up to 12 d (Laporte et al., 2016).

Despite evidence for the presence of PCs and MTs in plants and (some) macroalgae and their likely involvement in metal detoxification, few studies have assessed the relationship between these two thiol groups and any coordinated response to accumulate and detoxify the cellular contents of metals. However, some insight has been gained from recent investigations that assessed the overexpression of PCS and MT genes under metal exposure in different plant species, using RT-PCR amplification techniques (Zhang et al., 2005; González-Mendoza et al., 2007; Chaabene et al., 2018). The results showed differences between metals and with length of exposure. To address this issue for the first time in marine macroalgae, we have analyzed the production of GSH and PCs and the level of transcripts encoding MTs in *U. compressa*, in response to increasing intracellular concentrations of copper for up to 12 d exposure. Additionally, we assessed their possible role in the export of Cu ions to the external culture medium.

2. Materials and methods

2.1. Sampling of algae and water collection

The green macroalga *U. compressa* was collected, during spring, autumn and winter 2017, from the high intertidal zone at Cachagüa (32° 34′S), a site in central Chile with no history of metal contamination. Algal samples were transported to the laboratory in sealed plastic bags inside a cool-box. In the laboratory, material was rinsed three times with filtered seawater, cleaned manually and sonicated twice for 2 min using an ultrasound bath (HiLab Innovation Systems, model SK221OHP) to remove epiphytes. The algae were maintained in aerated seawater under an irradiance of 50 molμ m^{-2} s^{-1} on a photoperiod of 14 h light: 10 h dark, at 14 °C for 4 days, prior to experimentation. Seawater, was obtained from Quintay (33° 12′S), a pristine site in central Chile, prior to experimentation and filtered through 0.45 and 0.2 μm pore size membranes and stored in darkness at 4 °C.

2.2. Experimental protocols

In an initial experiment, we investigated temporal changes in the concentrations of intracellular Cu in the alga exposed to increasing concentrations of copper. Five g fresh weight (FW) of algae were transferred to individual acid-washed glass flasks containing 400 mL of seawater and one of five nominal copper (as $CuCl_2$) concentrations (0, 2.5, 5, 7.5 and 10 μM) and cultured for up to 12 d, in triplicates. To avoid the depletion of Cu and macronutrients, the culture medium was replenished every 48 h. The algae were harvested at 0, 1, 3, 5, 7, 9 and 12 days of experiments and washed twice with 100 mL of 50 mM Tris-10 mM EDTA for 20 min to remove excess copper bound to cell wall (Hassler et al., 2004). Samples from each replicate were dried to constant weight (approx. 0.6 g dry weight per flask) in an oven at 50 °C for 2 d and stored until copper level analysis.

In a second experiment, temporal changes in GSH and PCs levels as well as in the levels of MT transcripts were analyzed. One g of FW of

algae were transferred to individual acid-washed glass flasks containing 400 mL of seawater and one of five nominal copper concentrations (0, 2.5, 5, 7.5 and 10 μM) and cultured for up to 12 d, in triplicate. To avoid the depletion of Cu and macronutrients, the culture medium was replenished every 48 h. The algae were harvested at 0, 1, 3, 5, 7, 9 and 12 days of experiments and washed twice with 100 mL of 50 mM Tris-10 mM EDTA for 20 min to remove excess Cu bound to cell wall. Samples were immediately analyzed for GSH and PCs and those for MTs transcript analysis were rapidly frozen in liquid nitrogen and stored in a freezer at - 80 °C.

In a final experiment, the release of copper, GSH and PCs from cells following transfer to a copper-free medium was assessed. Five g of the alga were grown in 75 mL of seawater containing either 0 or 10 μM Cu for 5 d, with three replicates per treatment. At the end of the exposure period, the alga was washed with 100 mL of 50 mM Tris-10 mM EDTA, then transferred to flasks containing 75 mL of synthetic seawater (35 g of Sea Salts; Sigma-Aldrich, USA in 1 L of distilled water), with no added copper and cultured for up to 3 d. Triplicate samples per treatment were harvested on days 1, 2 and 3 d and then 4 g sub-samples were oven-dried and the remaining 1 g frozen in liquid nitrogen and stored, as before, to await analyses of copper and GSH/PCs, respectively. At the start of the post-exposure period and on each subsequent day of harvesting, samples of culture medium were collected and stored in a refrigerator to await analysis of total dissolved copper.

2.3. Quantification of intracellular copper and dissolved copper

Intracellular copper concentrations were determined as described in Valdés et al. (2017). Dried algae (0.5 g) were pre-digested overnight with 6 mL of 65% nitric acid (Merck) and 2 mL of 30% hydrogen peroxide (Merck) in teflon vials. Then, algal material was digested using a microwave oven (Milestone, model Ethos Easy) according to the intervallic program: 10 min at 200 °C with 1800 W power; 20 min at 200 °C for 20 min with 1800 W power; 20 min at 200 °C with 1800 W power. Digests were cooled to room temperature for 30 min, then diluted to 25 mL with ultrapure water. Quantification of copper was by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Perkin-Elmer, model ICP Optima 2000 DV). To account for precision and accuracy, copper concentrations were determined in certified reference material of the macroalga *U. lactuca* (BCR279), as above. Samples of growth medium were filtered through a 0.2 μm membrane and directly measured by the ICP-OES as described above.

2.4. Quantification of glutathione and phytochelatins

Concentrations of GSH and PCs were quantified following the protocol of Lavoie et al. (2009), with modifications. Briefly, 0.2 g of frozen *U. compressa* were ground to a powder with liquid nitrogen and extracted in a volume of 1.2 mL of 0.1% (w/v) trifluoroacetic acid (TFA) containing 6.3 mM diethylenetriamine-pentaacetic acid (DTPA). The cell mixture was centrifuged at 7.400 g for 20 min at 4 °C and the supernatant recovered and filtered through a 0.45 μm pore size membrane. Derivatization of thiol groups was performed by mixing 250 μL of the clear homogenate with 45 μL of 200 mM HEPES pH 8.2–6.3 mM DTPA and 1 μL of 25 mM monobromobimane (mBrB; Invitrogen, Oregon, USA) and the mixture then incubated in darkness at room temperature for 30 min. The derivatization process was stopped by the addition of 30 μL of metanesulphonic acid (MSA).

GSH and PCs were analyzed following the protocol of Mellado et al. (2012), using High Performance Liquid Chromatography (HPLC) with an Agilent 1260 Infinity platform; data were compiled using OpenLab CDS software. GSH and PCs (20 μL) were separated on a reverse phase C-18 column at 25 °C, eluted with a 0.1% TFA aqueous solution (solvent A) and 100% acetonitrile (solvent B) using a gradient (10 min from 0 to 20% channel B, 30 min from 20 to 35% and 10 min from 35 to 100% of solvent B), a flow rate of 1 mL min^{-1} and 200 bars of pressure. PCs were detected by fluorescence at 380 nm excitation and 470 nm emission wavelengths. Pure PCs with degrees of polymerization from n = 2–4 (AnaSpec Inc., San Jose, CA, USA) were dissolved in filtered water and used as standards. Retention time of GSH was 9.6 min and those of PC2, PC3 and PC4 were 12.7, 14.7 and 16.7 min, respectively.

2.5. Extraction of total RNA and quantification of MT transcripts

One hundred μg of frozen biomass were pulverized in a mortar with liquid nitrogen, to which 1 mL of Trizol reagent (Life Technologies, CA, USA) was added and homogenized until thawed. The mixture was centrifuged at 12.000 g for 10 min at 4 °C, and the supernatant recovered. Chloroform (200 μL) was added and vortexed for 10 s and the mixture left at room temperature for 3 min. The solution was centrifuged at 12.000 g for 15 min at 4 °C and the aqueous phase recovered. Isopropanol (500 μL) was added and the mixture then incubated for 10 min at room temperature. The solution was centrifuged at 12.000 g for 10 min at 4 °C, and the supernatant removed. The pellet was washed with 1 mL of 75% ethanol, gently vortexed and centrifuged at 7.000 g for 5 min at 4 °C. The ethanol phase was removed, the pellet dried for 15 min at room temperature and then dissolved in 50 μL of ultrapure water treated with DEPC and incubated for 10 min at 60 °C. Total RNA was quantified using a Quanti-iT Ribogreen RNA assay kit (Invitogen, USA) and verified using the absorbance ratio of A260/A280 and agarose gel electrophoresis, according to the manufacturer's instructions, and then stored at −80 °C.

Quantification of the relative levels of MT transcripts was performed by qRT-PCR using a real time thermocycler Rotorgene 6000 (Corbett, Australia). Reactions were performed using One-step kit (Bioline, UK), with 75 ng of total RNA, 400 nM of PCR primers, and 3 mM magnesium chloride. *U. compressa* β-tubulin was used as a housekeeping gene since its levels do not change under copper excess (Le Bail et al., 2008). PCR primers were designed using Primer Blast online software (NCBI, USA) based on the transcriptome of *U. compressa* (Laporte et al., 2016) and were: TUB-F: 5′TGCAACTTTTGTAGGCAACTC3′; TUB-R: 5′CAGTGAACTCCATCTCGTCC3′; MT3-F: 5′CCAGTGCCAAACCGAAGATG3′; MT3-R: 5′TGCTAGCAGGCACAGTCGTC3′; MT4-F: 5′AACTGCGACTGTGCTGACCA3′; MT4-R: 5′CAGTCTCGATGTGGCTCTTCT3′; MT6-F: 5′GCACTCCTGAGACCTGCACT3′; MT6-R: 5′ATCCTTCGCGGGTGAGCAAG3′; MT7-F. 5′TCTTGTTGTGAAGCCAGTGA3′; MT7-R: 5′CACAGTTGCATTCTGCGGTT3′. Amplification was performed for 5 s at 95 °C and 10 s at 54 °C for MTs, for 10 s at 50 °C for tubulin and for 5 s at 60 °C and 40 cycles of amplification for all. The relative levels of MT transcripts are expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.6. Statistical analyses

Statistical analyses were performed with the Prism 6 statistical package (Graph Pad Software Inc., California, USA). Following confirmation of normality and homogeneity of variance, significant differences between treatments were determined by two-way ANOVA and Tukey's multiple comparison post-hoc test, at a 95% confidence interval.

3. Results

3.1. Effect of increasing concentrations of copper on intracellular copper levels

The concentrations of intracellular copper in the algae cultivated under different external copper concentrations and exposure periods are presented in Fig. 1. Prior to exposure, the algae had a mean baseline intracellular copper concentration of 10 μg g^{-1} DW (Fig. 1A). By the end of the experiment, intracellular concentrations had increased to 164, 346, 426, and 620 μg g^{-1} DW on exposure to 2.5, 5, 7.5 and 10 μM Cu, respectively (Fig. 1A). Over the 12 d period of exposure, there was a

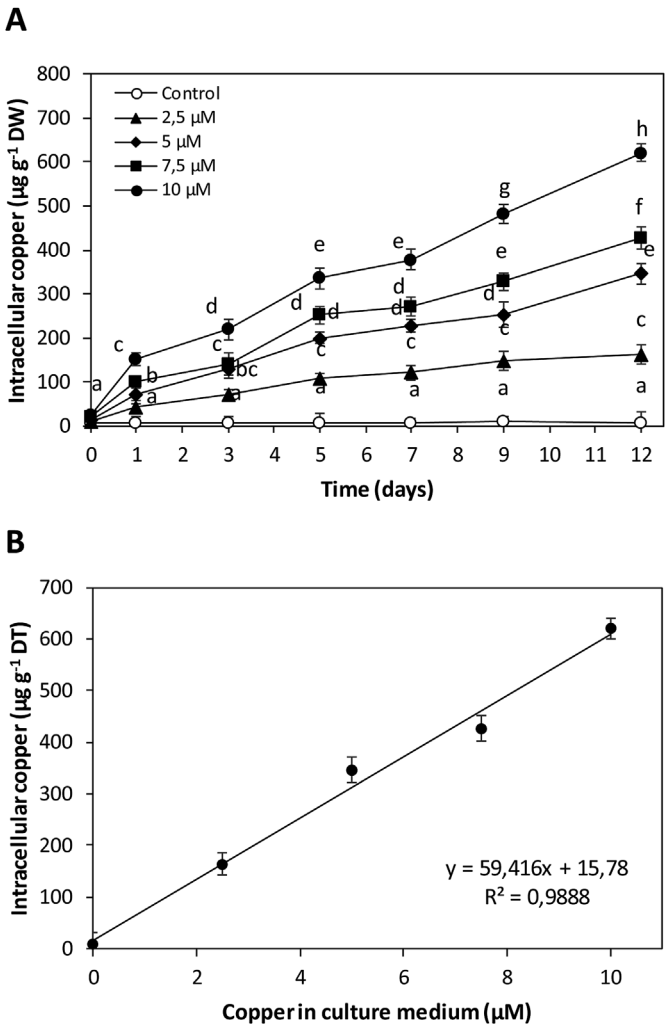

Fig. 1. Concentrations (µg g^{-1} DW) of intracellular copper (A) in *U. compressa* cultured in seawater without copper addition (control, open circles), with 2.5 µM (lack triangles), 5 µM copper (black diamonds), 7.5 µM copper (black squares) and 10 µM copper (black circles) for 0–12 days. Correlation between intracellular copper accumulation and the level of copper exposure. Symbols represent mean values of three replicates. ± SD.

linear relationship between intracellular copper and external concentrations ($R^2 = 0.99$) (Fig. 1B). The intracellular concentrations corresponded to around 40% of total copper added to cultures for each copper concentration.

3.2. Effect of increasing concentrations of copper on GSH and PC levels

The concentrations of GSH measured in the algae exposed to a range of external copper concentrations for time periods of up to 12 d are shown in Fig. 2. At day 0 control samples showed 17 nmol g^{-1} FW GSH, whereas in copper treated algae GSH levels were always significantly lower than controls (Fig. 2A). Between days 1 and 12, general trends on GSH showed increasing concentrations up to 5 µM copper, and then decreasing with greater levels of copper exposure (Fig. 2B, C, D). Apart from day 5, GSH levels at the highest copper exposure (10 µM) were lower than controls (Fig. 2B, C, D).

In terms of PCs, only PC2 and PC4 were detected. With PC2, no significant differences were observed among treatments at day 0 (Fig. 3A), although samples collected later at 1, 5 and 12 days showed the highest levels of PC2 at the greatest copper exposures (7.5 and 10 µM) (Fig. 3B, C, D). Indeed, the highest PC2 levels were recorded over 700 nmol g^{-1} FW under 10 µM at day 12 (Fig. 3D). A similar trend was observed with PC4 (Fig. 3E, F, G, H), although the highest levels were detected over 80 nmol g^{-1} FW under 7.5 µM at day 1 (Fig. 3E). In addition, it is important to point out that there is a positive correlation ($R = 0.71$, $P < 0.0001$) among the level of thiols groups present in GSH and PCs subject to the levels of intracellular copper at day 12 of culture (Fig. 4).

3.3. Effect of increasing copper concentrations on MT transcript levels

The results of the effects of copper exposure on the expression levels of MTs are presented in Fig. 4. Three MTs genes, having similarities with animal MTs, were assessed in *U. compressa*: *Crassostrea virginica*-like *mt3*; the *Mytilus galloprovincialis*-like *mt6*; and the *Dressenia polymorpha*-like *mt7*. The expression of MT-genes under copper exposure was mostly higher than in controls but the pattern of increase varied between concentration and time-period of copper exposure. For *mt3*, even though the expression increased under all copper concentrations, there were no observable trends up to day 7 (Fig. 5A). However, at day 9 the relative expression of *mt3* was 9 and 15 times higher at 7.5 and 10 µM Cu, respectively, compared to controls (Fig. 5A). By day 12, *mt3* expression levels at 7.5 and 10 µM copper had decreased but were still significantly higher than in other copper treatments (Fig. 5A). The expression of *mt6*, increased until day 7 at 2.5 µM copper, but decreased thereafter to initial baseline levels by days 9 and 12 (Fig. 5B). Moreover, the expression of *mt6* oscillated almost proportionally to the levels of copper exposure, peaking at day 5 for 7.5 and 10 µM copper and day 7 for 5 µM copper, then declining at day 9 before finally reaching the highest expression at day 12 (Fig. 5B). The expression of *mt7* showed no obvious trends under 2.5, 5, and 7.5 µM copper, although there was a peak 4 times higher than controls at 7.5 µM copper on day 5 (Fig. 5C). In contrast, at 10 µM copper, the expression of *mt7* increased markedly (9-fold) for 3 days of exposure but then progressively decreased to almost baseline levels by day 12 of the experiment (Fig. 5C).

3.4. Release of copper ions and GSH, but not PCs, to the culture medium

In order to analyze whether *U. compressa* is able to release copper, GSH and/or PCs to the culture medium, the alga was cultivated with 10 µM copper for 5 days, then transferred to synthetic seawater, that contained no copper, and cultivated for 1, 2 and 3 d. The mean intracellular copper concentration in the algal material following exposure to 10 µM copper for 5 d was 1152 nmoles that decreased to 145 nmoles by day 3 (Fig. 6A). Concurrently, the concentration of copper in the medium increased from 0 to 47 nmol L^{-1} by day 3 (Fig. 6A). Surprisingly, the level of copper released to the culture medium was lower than the level of copper that remained inside the alga. In addition, the level of GSH in the growth medium increased from 0 to 46 nmoles by day 3 (Fig. 6B). Thus, the concentrations of copper ions and GSH in the growth media were similar by day 3. No PCs or small proteins were detected in the artificial seawater culture medium.

4. Discussion

In this work, we have shown that intracellular copper accumulation in the marine alga *U. compressa* directly correlates copper concentration in the culture medium, displaying a linear relationship over the exposure period. The concentration of copper within cells corresponds to around 40% of copper added to the growth medium for each copper concentration. This latter finding are in accordance with those obtained in the brown macroalga *E. siliculosus* cultivated with 1.6 and 2.4 µM copper for 10 d in which intracellular copper corresponds to around 50% of total copper added to the growth medium (Roncarati et al., 2015). This suggests that *U. compressa* may display more efficient mechanisms of copper detoxification or exclusion in the cell wall than *E. siliculosus* (see below). Moreover, the intracellular copper concentrations in the alga used in this study cultivated with 10 µM copper for 12

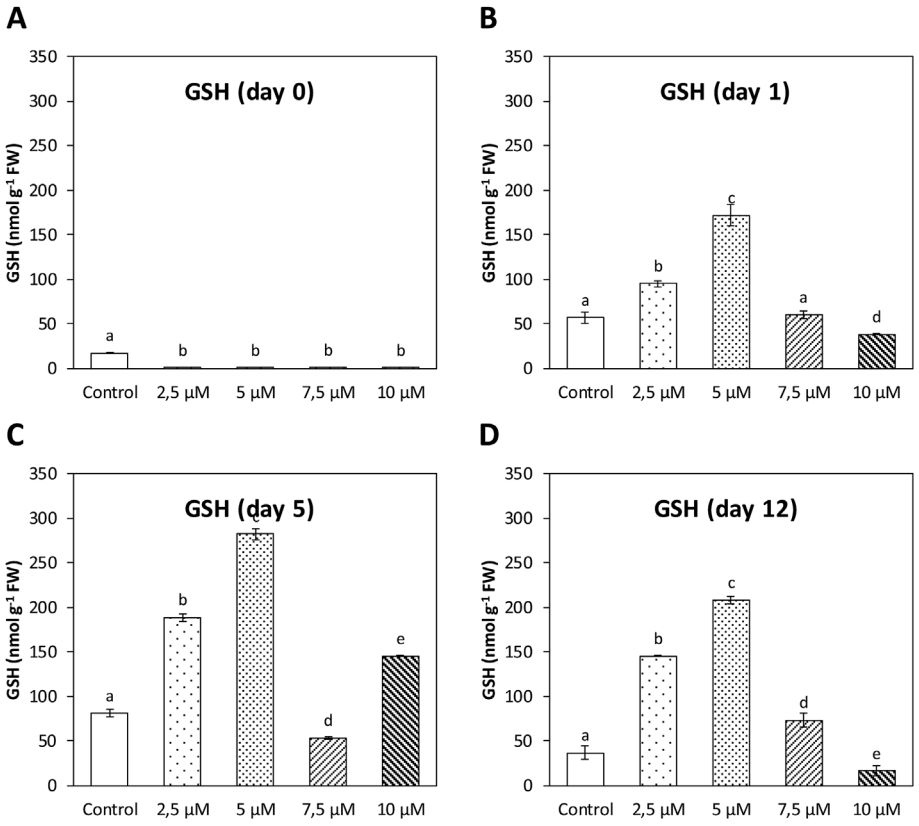

Fig. 2. Concentrations (nmol g^{-1} FW) of glutathione (GSH), in *Ulva compressa* cultivated seawater without copper addition (open circles), 2.5 µM copper (black triangles), 5 µM copper (black diamonds), 7.5 µM copper (black square), and 10 µM copper (black circles) at day 0 (A), 1 (B), 5 (C), and 12 (D). Symbols represent mean values of three replicates. ± SD.

days reached 620 µg g^{-1} DW (this work). Furthermore, when *U. compressa* was cultivated with 25 µM for 10 d the internal copper concentration was 864 ± 42 µg g^{-1} DW (data not shown). If the linear relationship had been maintained at this external concentration, the expected intracellular concentration would have been 1.500 µg g^{-1} DW. This result suggests that an extrusion mechanism is operating in *U. compressa* once the internal copper level reaches a threshold near 900 µg g^{-1} DW. Similar results were obtained with a copper-tolerant strain Es524 of *E. siliculosus* cultivated with 2.4 µM copper that shows a decrease intracellular copper at day 10 whereas the less-tolerant strain LIA did not show such a decrease suggesting that an extrusion mechanism is operating in Es524 (Roncarati et al., 2015). Thus, it is possible that marine macroalgae can extrude copper to the extracellular medium to counter its potential toxic effects. Evidence for this comes from our final experiment in which the alga was cultivated with 10 µM copper for 5 days and then incubated in synthetic seawater without copper for a further 3 days. There was a decrease in intracellular copper and concomitant increase in copper in the external medium indicating that an extrusion detoxification mechanism is operating in *U. compressa*. In this sense, it is important to mention that copper ions present in synthetic seawater correspond to Cu^{+2} as indicated by anodic dissolution voltammetry (data not shown).

Beyond day 0, levels of GSH increased with 2.5 and 5 µM copper, and then decreased. On the other hand, also after day 0, the levels of PCs increased mainly with the highest levels of copper exposure (7.5 and 10 µM). In addition, there was a significant correlation among the level of GSH and PCs upon intracellular copper accumulation, suggesting that these thiol-containing molecules may be involved in copper accumulation and detoxification in *U. compressa*. In this context, it has been shown that the overexpression of PCS of the macrophyte *Ceratophyllum demersum* in tobacco plants induced the accumulation of cadmium and arsenic (Shukla et al., 2012). Moreover, the overexpression of PCS of *A. thaliana* in tobacco induced cadmium and arsenic accumulation (Zanella et al., 2016). In contrast, no production of PCs was observed in *Medicago sativa* under copper excess, suggesting that PCs may not be involved in accumulation and detoxification in this species (Flores-Cáceres et al., 2015). The information suggests that the role of GSH and PCs in metal accumulation is an inter-specific mechanism among different macrophytes.

The increase in the relative levels of transcripts encoding three MTs, mainly at the highest concentrations of copper, is indicative of the involvement of MTs in copper accumulation. Interestingly, the increase in levels of MT7 occurred in the first 3 days of exposure whereas increases in MT3 and MT6 occurred later and particularly between days 9 and 12. Thus, the increase in MT expression appears to be a later response compare with the increase in GSH and PCs levels. This is the first evidence for the combined involvement of GSH/PCs and MTs in copper accumulation in a marine macroalga. However, our results are in accordance with those obtained in the black mangrove *Avicennia germinans* exposed to cadmium and copper that showed an increased expression of PCS after 4 h of exposure and only a slight increase in AvMT2 transcripts at 16 h of exposure (Gonzalez-Mendoza et al., 2007). It is important to mention that the three mentioned MTs have been cloned and expressed in bacteria leading to copper accumulation (Zúñiga et al., not yet published). Thus, expression of MTs in *U. compressa* may be also involved copper accumulation. Moreover, it is important to point out that the increase in GSH and PCs syntheses and MT expression were mostly transient which is in contrast with the linear increase in intracellular copper, at least up to an exposure concentration of 10 µM copper. Thus, it is possible that the coordinated responses and complementarity of GSH, PCs and MTs may account for the linear accumulation of intracellular copper in *U. compressa*.

On the other hand, the alga cultivated with 10 µM copper for 5 days and then incubated in artificial seawater for 3 days showed the release of copper ions and GSH to the extracellular medium. The latter is similar to what has been shown in several other micro and macroalgae, where cysteine and/or GSH have been observed to be exudate under exposure to metal excess, including species of the genus *Ulva* under

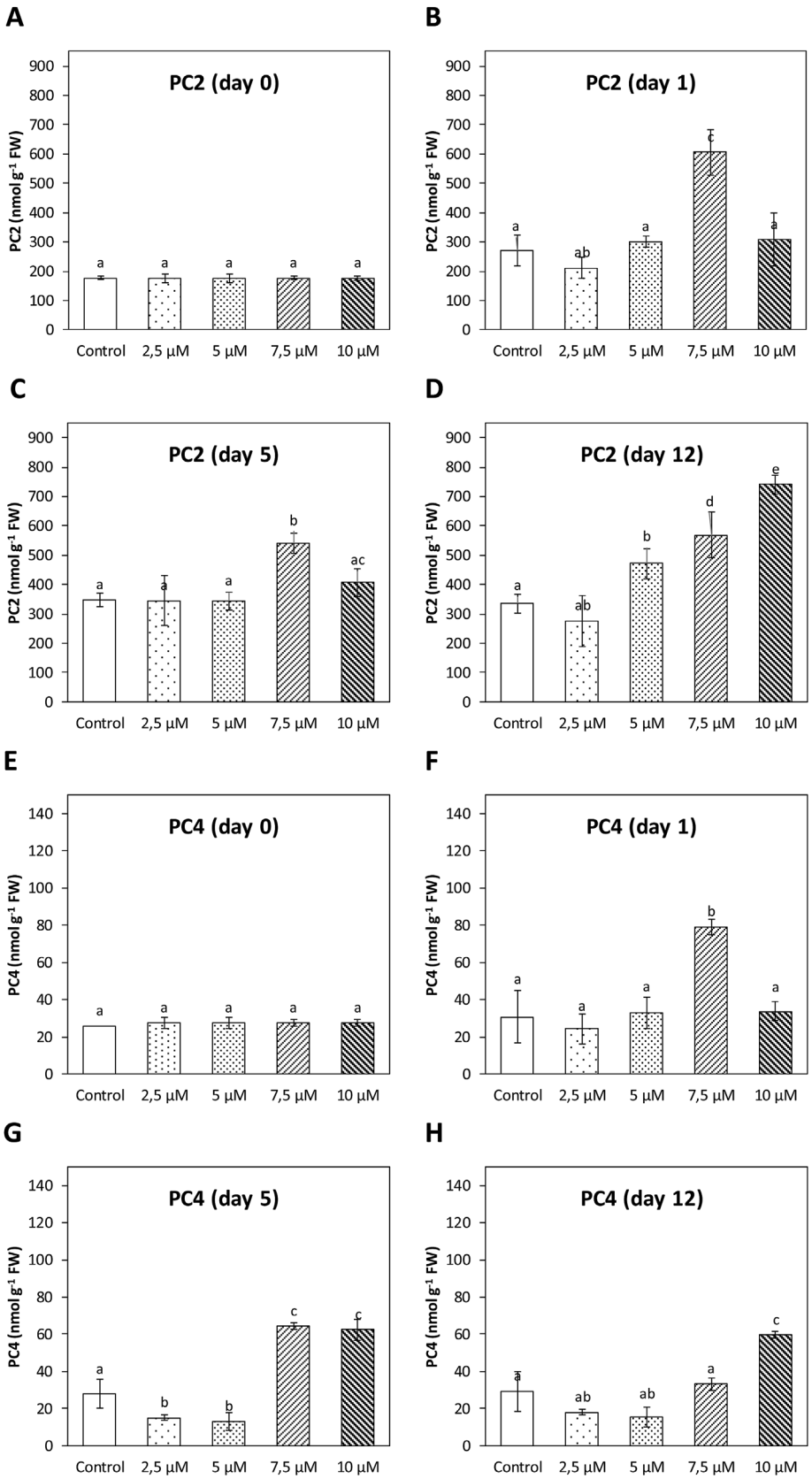

Fig. 3. Concentrations (nmol g^{-1} FW) of PCs in *Ulva compressa* cultivated seawater without copper addition (open circles), 2.5 µM copper (black triangles), 5 µM copper (black diamonds), 7.5 µM copper (black square), and 10 µM copper (black circles). PC2 at day 0 (A), 1 (B), 5 (C), and 12 (D), and PC4 at day 0 (E), 1 (F), 5 (G), and 12 (H). Symbols represent mean values of three replicates. ± SD.

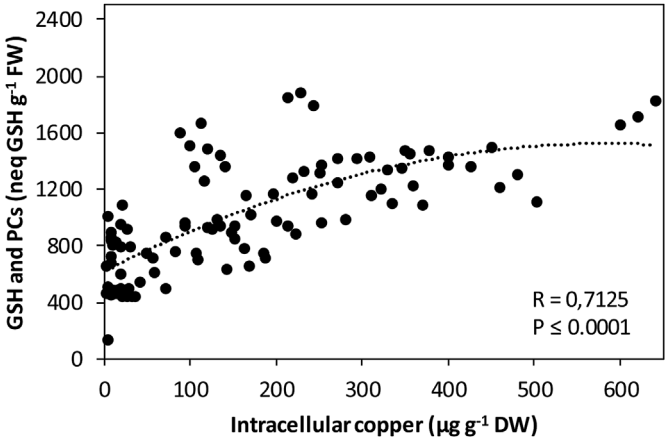

Fig. 4. Correlation among mean values of intracellular copper accumulation and intracellular GSH and PCs content expressed as nanoequivalents of GSH.

increased copper levels (e.g. Leal et al., 1999, Vasconcelos and Leal, 2001a, b, Vasconcelos et al., 2002, Vasconcelos and Leal, 2008). These authors have suggested that the exudation of these metal ligands can complex metals in the extracellular medium, reducing their bioavailability and capacity to cross the cell wall and membrane towards the intracellular media. The exudation of other ligands to reduce external metal bioavailability is a phenomena that has been described in several other macroalgae species; for instance, in brown seaweeds that have shown exudation of polyphenolic compounds in response to copper excess (e.g. Gledhill et al., 1999; Connan and Stengel, 2011). The loss of copper ions from cells can be also mediated by copper transporters such as those described in *Silene vulgaris* roots (Van Hoof et al., 2001) and cucumber and maize roots which are ATP-dependent (Burzynski and Kolano, 2003) but do not require GSH. In addition, the level of copper detected in the growth medium is lower than the concentrations lost from inside the cells. The latter may result from the trapping of copper ions in the cell wall that is removed by washing with Tris-EDTA before moving the alga to artificial seawater. Moreover, no PCs or small peptides or proteins were detected in the culture medium (data not shown). Thus, the alga may exudate GSH in order to reduce the bioavailability of released copper.

In conclusion, accumulation of intracellular copper linearly correlates with increasing concentrations of copper in culture medium and with elapsed time of culture. The intracellular increase in levels of GSH, PCs levels and MTs may be involved in copper accumulation and detoxification. Interestingly, GSH, PCs and MTs were induced at different times within the duration of copper exposure experiments, suggesting a coordinated and complementary role between these ligands as an accumulation and detoxification mechanisms. In addition, the alga released copper ions indicating an exclusion mechanism to avoid copper excess in the intracellular media. Finally, it was observed the exudation of GSH to the extracellular medium in *U. compressa*, suggesting its role complexing external excess of bioavailable copper.

CRediT authorship contribution statement

Axel Navarrete: Formal analysis, Data curation. **Melissa Gómez:** Formal analysis, Data curation. **Rodrigo A. Contreras:** Formal analysis, Data curation. **Patricia Díaz:** Formal analysis, Data curation. **Gabriela Lobos:** Formal analysis, Data curation. **Murray T. Brown:** Writing – original draft. **Claudio A. Sáez:** Writing – original draft. **Alejandra Moenne:** Writing – original draft.

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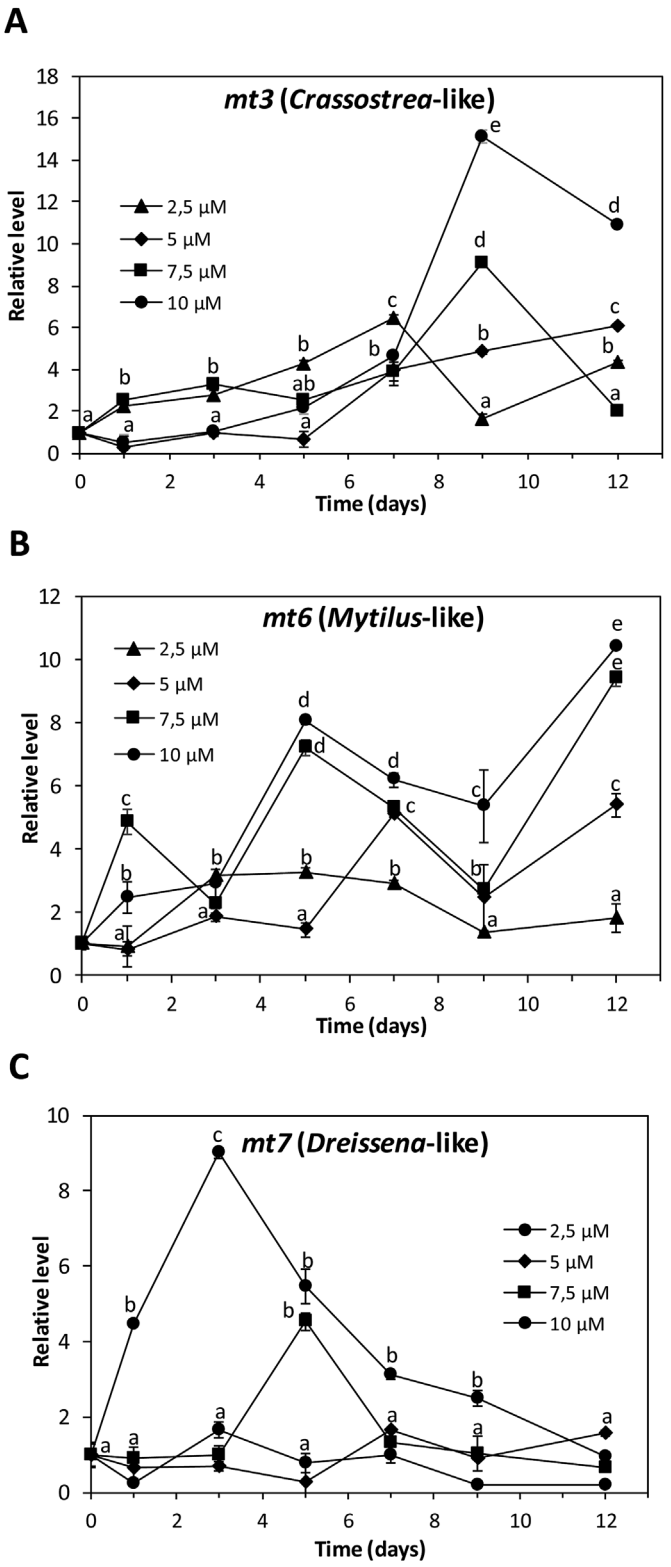

Fig. 5. Relative levels of transcripts encoding metallothioneins (MTs) in *Ulva compressa* cultivated in seawater with 2.5 µM (black triangles), 5 µM (black diamonds), 7.5 µM (black squares) and 10 µM copper (black circles) for 0–12 days. MTs correspond to *mt3* encoding a *Crassostrea virginica*-like MT (A), *mt6* encoding *Mytilus edulis*-like MT (B) and *mt7* encoding a *Dreissena polymorpha*-like MT (C). The relative level of MTs is expressed as $2^{-\Delta\Delta CT}$. Symbols represent mean values of three replicates ± SD.

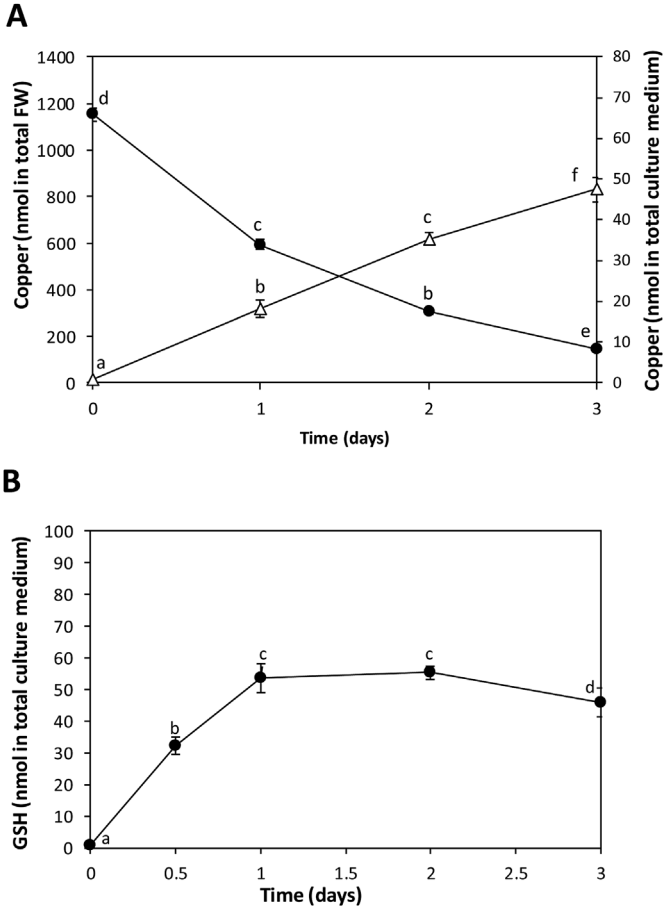

Fig. 6. Concentrations (μg g^{-1} DW) of intracellular copper in *Ulva compressa* cultivated in seawater with 10 μM copper for 5 days and then transferred to synthetic seawater for 3 days (black circles, A). Concentrations of copper (open triangles, A) and GSH (black circles, B) in synthetic seawater. Symbols represent mean values of three replicates ± SD.

11160369 to C.A.S.

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